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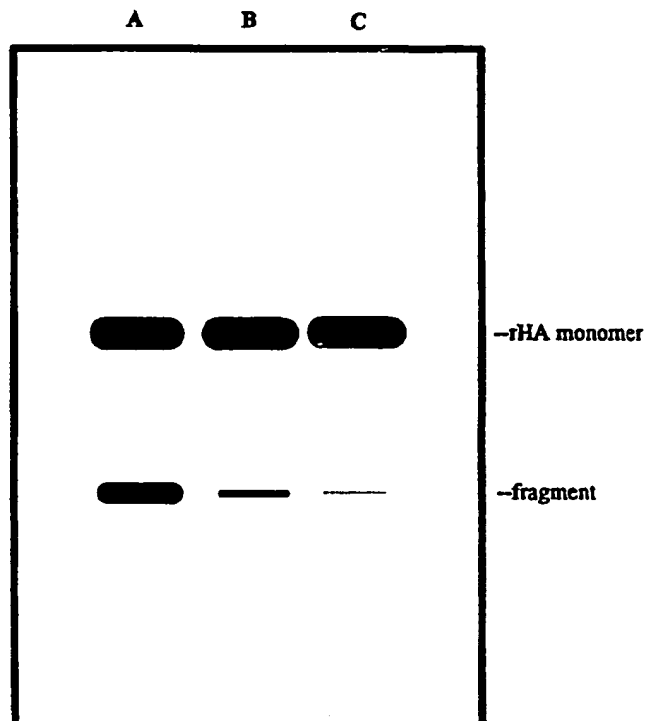
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(54) Title: YEAST STRAINS AND MODIFIED ALBUMINS

(57) Abstract

Albumin, for example human albumin, is expressed and secreted in yeast which has been mutated to lack the yeast aspartyl protease 3 (Yap3p) or its equivalent, thereby reducing the production of a 45kD albumin fragment. A further reduction is achieved by additionally deleting the Kex2p function. Alternatively, a modified albumin is prepared which is not susceptible to Yap3p cleavage, for example human albumin which is R410A, K413Q and K414Q.



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YEAST STRAINS AND MODIFIED ALBUMINS

Field of the invention

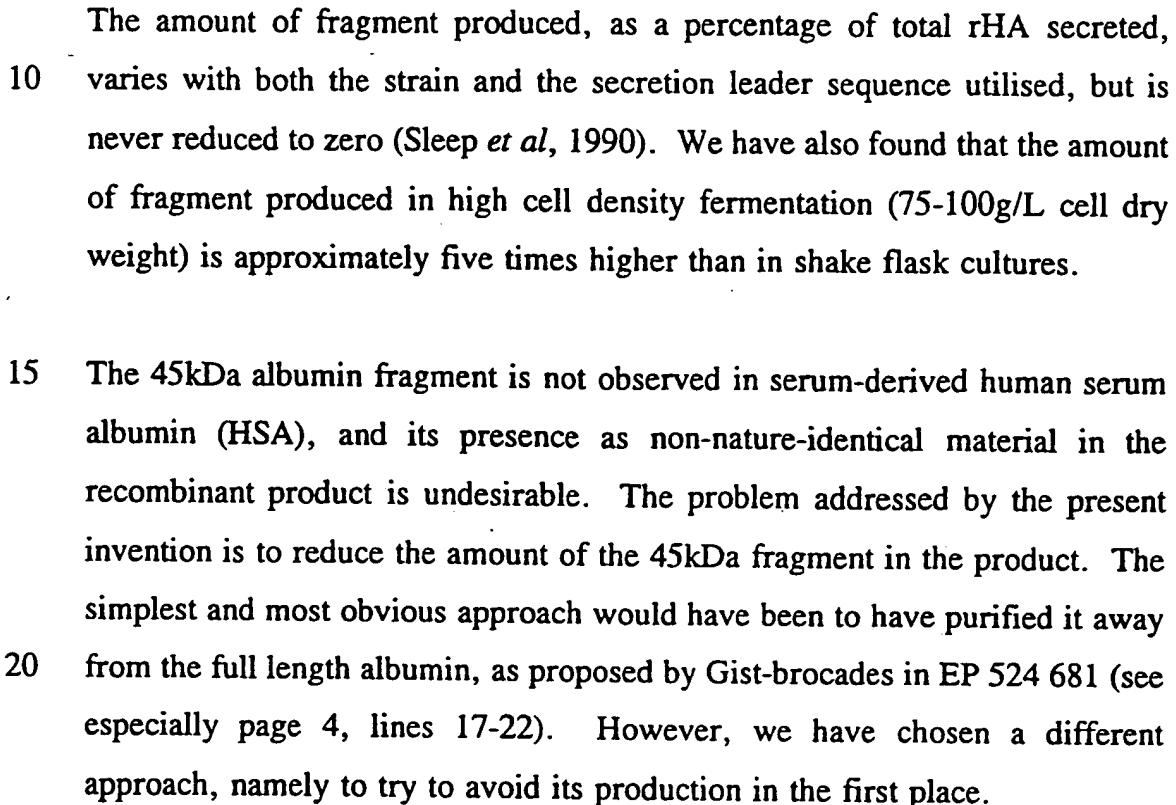
- 5 The present invention relates to the production of recombinant human albumin (rHA) by yeast species.

Background and prior art

- 10 Human serum albumin (HSA) is a protein of 585 amino acids that is responsible for a significant proportion of the osmotic pressure of serum, and also functions as a carrier of endogenous and exogenous ligands. It is used clinically in the treatment of patients with severe burns, shock, or blood loss, and at present is produced commercially by extraction from human blood. The
15 production of recombinant human albumin (rHA) in microorganisms has been disclosed in EP 330 451 and EP 361 991.

- In recent years yeast species have been widely used as a host organisms for the production of heterologous proteins (reviewed by Romanos *et al*, 1992),
20 including rHA (Sleep *et al*, 1990, 1991; Fleer *et al*, 1991). Yeasts are readily amenable to genetic manipulation, can be grown to high cell density on simple media, and as eukaryotes are suitable for production of secreted as well as cytosolic proteins.

- 25 When *S. cerevisiae* is utilised to produce rHA, the major secreted protein is mature 67kDa albumin. However, a 45kDa N-terminal fragment of rHA is also observed (Sleep *et al*, 1990). A similar fragment is obtained when rHA is expressed in *Kluyveromyces* sp. (Fleer *et al*, 1991) and *Pichia pastoris* (EP 510 693). The fragment has the same N-terminal amino acid sequence as
30 mature rHA, but the carboxy terminus is heterogeneous and occurs between



Sleep *et al* (1990) postulated that rHA fragment is produced within the cell and is not the result of extra-cellular proteolysis. These authors codon-optimised the HSA cDNA from Glu³⁸² to Ser⁴¹⁹ but this had no effect on production of rHA fragment. They noted that a potential Kex2p processing site in the rHA amino acid sequence, Lys⁴¹³Lys⁴¹⁴, is in close proximity to the heterogeneous carboxy terminus of the fragment, but neither use of a *kex2* host strain (ie a strain harbouring a mutation in the *KEX2* gene such that it does not produce the Kex2p protease), nor removal of the potential cleavage site by site-directed

mutagenesis of the codon for Lys⁴¹⁴, resulted in reduction in the amount of the fragment.

There is a vast array of yeast proteases which could, in principle, be degrading
 5 a desired protein product, including (in *S. cerevisiae*) yscA, yscB, yscY, yscS, other vacuolar proteinases, yscD, yscE, yscF (equivalent to kex2p), ysc α , yscIV, yscG, yscH, yscJ, yscE and kex1.

Bourbonnais *et al* (1991) described an *S. cerevisiae* endoprotease activity
 10 specific for monobasic sites, an example of which (Arg⁴¹⁰) exists in this region of albumin. This activity was later found to be attributable to yeast aspartyl protease 3 (Yap3) (Bourbonnais *et al*, 1993), an enzyme which was originally described by Egel-Mitani *et al* (1990) as an endoprotease similar to Kex2p in specificity, in that it cleaved at paired basic residues. Further work suggested
 15 that Yap3p is able to cleave monobasic sites and between, and C-terminal to, pairs of basic residues, but that cleavage at both types of sites is dependent on the sequence context (Azaryan *et al*, 1993; Cawley *et al*, 1993).

As already discussed, the region of the C-terminus of rHA fragment contains
 20 both a monobasic (Arg⁴¹⁰) and a dibasic site (Lys⁴¹³Lys⁴¹⁴). However, even though a Kex2p-like proteolytic activity is present in human cells and is responsible for cleavage of the pro sequence of HSA C-terminal to a pair of arginine residues, the fragment discussed above is not known to be produced in humans. This indicates that the basic residues Arg⁴¹⁰, Lys⁴¹³ and Lys⁴¹⁴ are
 25 not recognised by this Kex2p-like protease, in turn suggesting that this region of the molecule may not be accessible to proteases in the secretory pathway. Thus, the Yap3p protease could not have been predicted to be responsible for the production of the 45kDa fragment. In addition, Egel-Mitani *et al* (1990 *Yeast* 6, 127-137) had shown Yap3p to be similar to Kex2p in cleaving the
 30 MF α propheromone. Since removal of the Kex2p function alone does not

reduce the amount of the fragment produced, there was no reason to suppose that removal of the Yap3p function would be beneficial. Indeed, Bourbonnais *et al* (1993) showed that *yap3* strains had a decreased ability to process pro-somatostatin, and therefore taught away from using *yap3* strains in the
5 production of heterologous proteins.

Summary of the invention

The solution to the problem identified above is, in accordance with the
10 invention, to avoid or at least reduce production of the fragment in the initial fermentation, rather than to remove it during purification of the albumin. We have now found that, out of the 20 or more yeast proteases which are so far known to exist, it is in fact the Yap3p protease which is largely responsible for the 45kD fragment of rHA produced in yeast. The present invention provides
15 a method for substantially reducing the amount of a 45kDa fragment produced when rHA is secreted from yeast species. The reduction in the amount of fragment both improves recovery of rHA during the purification process, and provides a higher quality of final product. A further, and completely unexpected, benefit of using *yap3* strains of yeast is that they can produce 30-
20 50% more rHA than strains having the Yap3p function. This benefit cannot be accounted for merely by the reduction of rHA fragment from ~15% to 3-5%.

Thus, one aspect of the present invention provides a process for preparing albumin by secretion from a yeast genetically modified to produce and secrete
25 the albumin, comprising culturing the yeast in a culture medium such that albumin is secreted into the medium, characterised in that the yeast cells have a reduced level of yeast aspartyl protease 3 proteolytic activity.

Preferably, the said proteolytic activity is an endoprotease activity specific for
30 monobasic sites and for paired basic amino acids in a polypeptide.

Suitably, the yeast is *S. cerevisiae* which lacks a functional *YAP3* gene. However, the invention is not limited to the use of *S. cerevisiae*, since the problem of 45 kDa fragment production is found also in other yeast genera, for example *Pichia* and *Kluyveromyces*, which shows that they have equivalent proteases (ie Yap3p proteolytic activity); see Clerc *et al* (1994), page 253. We have confirmed this by hybridisation analysis to locate homologues of Yap3p in non-*Saccharomyces* genera. A gene is regarded as a homologue, in general, if the sequence of the translation product has greater than 50% sequence identity to Yap3p. In non-*Saccharomyces* genera, the Yap3p-like protease and its gene may be named differently, but this does not of course alter their essential nature.

The level of fragment can be reduced still further if, as well as substantially eliminating the Yap3p proteolytic activity, the Kex2p function is also substantially eliminated even though, as mentioned above, elimination of the Kex2p function alone does not affect the level of fragment. As in the case of Yap3p, the Kex2p function is not restricted to *Saccharomyces*; see Gellissen *et al* (1992), especially the sentence bridging pages 415 and 416, showing that *Pichia* has a Kex2p function. The genes encoding the Kex2p equivalent activity in *Kluyveromyces lactis* and *Yarrowia lipolytica* have been cloned (Tanguy-Rougeau *et al*, 1988; Enderlin & Ogrydziak, 1994).

A suitable means of eliminating the activity of a protease is to disrupt the host gene encoding the protease, thereby generating a non-reverting strain missing all or part of the gene for the protease (Rothstein, 1983). Alternatively, the activity can be reduced or eliminated by classical mutagenesis procedures or by the introduction of specific point mutations by the process of transplacement (Winston *et al*, 1983). Preferably, the activity of the enzyme is reduced to at most 50% of the wild-type level, more preferably no more than 25%, 10% or 5%, and most preferably is undetectable. The level of Yap3p proteolytic

activity may be measured by determining the production of the 45 kDa fragment, or by the ^{125}I - β_{h} -lipoprotein assay of Azaryan *et al* (1993), also used by Cawley *et al* (1993). Kex2p proteolytic activity may similarly be measured by known assays, for example as set out in Fuller *et al* (1989).

5

The albumin may be a human albumin, or a variant thereof, or albumin from any other animal.

By "variants" we include insertions, deletions and substitutions, either
10 conservative or non-conservative, where such changes do not substantially alter the oncotic, useful ligand-binding or non-immunogenic properties of albumin. In particular, we include naturally-occurring polymorphic variants of human albumin; fragments of human albumin which include the region cleaved by Yap3p, for example those fragments disclosed in EP 322 094 (namely HSA (1-
15 n), where n is 369 to 419) which are sufficiently long to include the Yap3p-cleaved region (ie where n is 403 to 419); and fusions of albumin (or Yap3p-cleavable portions thereof) with other proteins, for example the kind disclosed in WO 90/13653.

20 By "conservative substitutions" is intended swaps within groups such as Gly, Ala; Val, Ile, Leu; Asp, Glu; Asn, Gln; Ser, Thr; Lys, Arg; and Phe, Tyr.

Such variants may be made using the methods of protein engineering and site-directed mutagenesis as described below.

25

A second aspect of the invention provides a modified albumin having at least 90% sequence identity to a naturally-occurring albumin, which naturally-occurring albumin is susceptible to cleavage with the *S. cerevisiae* yeast aspartyl protease 3 (Yap3p) when expressed in yeast, characterised in that the
30 modified albumin is not susceptible to such cleavage.

Preferably, the modified albumin lacks a monobasic amino acid present in the naturally-occurring albumin protein. Suitably, the said monobasic amino acid is arginine. Conveniently, the modified albumin additionally lacks a pair of basic amino acids present in the naturally-occurring albumin, especially any of Lys, Lys; Lys, Arg; Arg, Lys; or Arg, Arg. Thus, in one particular embodiment, the naturally-occurring albumin is human albumin and the modified protein lacks Arg⁴¹⁰ and, optionally, one or both Lys⁴¹³Lys⁴¹⁴ lysines. For example, the modified albumin may be human albumin having the amino acid changes R410A, K413Q, K414Q. Equivalent modifications in bovine serum albumin include replacing the Arg⁴⁰⁸ and/or one or both of Arg⁴¹¹Lys⁴¹². The person skilled in the art will be able to identify monobasic sites and pairs of basic residues in other albumins without difficulty.

The numbering of the residues corresponds to the sequence of normal mature human albumin. If the albumin is a variant (for example a polymorphic form) having a net deletion or addition of residues N-terminal to the position identified, then the numbering refers to the residues of the variant albumin which are aligned with the numbered positions of normal albumin when the two sequences are so aligned as to maximise the apparent homology.

A third aspect of the invention provides a polynucleotide encoding such a modified albumin.

The DNA is expressed in a suitable yeast (either the DNA being for a modified albumin, or the yeast lacking the Yap3p function) to produce an albumin. Thus, the DNA encoding the albumin may be used in accordance with known techniques, appropriately modified in view of the teachings contained herein, to construct an expression vector, which is then used to transform an appropriate yeast cell for the expression and production of the albumin.

The DNA encoding the albumin may be joined to a wide variety of other DNA sequences for introduction into an appropriate host. The companion DNA will depend upon the nature of the host, the manner of the introduction of the DNA into the host, and whether episomal maintenance or integration is desired.

5

Generally, the DNA is inserted into an expression vector, such as a plasmid, in proper orientation and correct reading frame for expression. The vector is then introduced into the host through standard techniques and, generally, it will be necessary to select for transformed host cells.

10

Host cells that have been transformed by the recombinant DNA of the invention are then cultured for a sufficient time and under appropriate conditions known to those skilled in the art in view of the teachings disclosed herein to permit the expression and secretion of the albumin, which can then be recovered, as is known.

15

Useful yeast plasmid vectors are pRS403-406 and pRS413-416 and are generally available from Stratagene Cloning Systems, La Jolla, CA 92037, USA. Plasmids pRS403, pRS404, pRS405 and pRS406 are Yeast Integrating plasmids (YIps) and incorporate the yeast selectable markers *HIS3*, *TRP1*, *LEU2* and *URA3*. Plasmids pRS413-416 are Yeast Centromere plasmids (YCps). Other yeast expression plasmids are disclosed in EP-A-258 067, EP-A-286 424 and EP-A-424 117.

20

25 The polynucleotide coding sequences encoding the modified albumin of the invention may have additional differences to those required to produce the modified albumin. For example, different codons can be substituted which code for the same amino acid(s) as the original codons. Alternatively, the substitute codons may code for a different amino acid that will not affect the activity or immunogenicity of the albumin or which may improve its activity

30

or immunogenicity, as well as reducing its susceptibility to a Yap3p protease activity. For example, site-directed mutagenesis or other techniques can be employed to create single or multiple mutations, such as replacements, insertions, deletions, and transpositions, as described in Botstein and Shortle
 5 (1985). Since such modified coding sequences can be obtained by the application of known techniques to the teachings contained herein, such modified coding sequences are within the scope of the claimed invention.

Exemplary genera of yeast contemplated to be useful in the practice of the
 10 present invention are *Pichia*, *Saccharomyces*, *Kluyveromyces*, *Candida*, *Torulopsis*, *Hansenula* (now reclassified as *Pichia*), *Histoplasma*, *Schizosaccharomyces*, *Citeromyces*, *Pachysolen*, *Debaromyces*, *Metschnikowia*, *Rhodospiridium*, *Leucosporidium*, *Botryoascus*, *Sporidiobolus*, *Endomycopsis*, and the like. Preferred genera are those selected from the group consisting of
 15 *Pichia*, *Saccharomyces*, *Kluyveromyces*, *Yarrowia* and *Hansenula*. Examples of *Saccharomyces* sp. are *S. cerevisiae*, *S. italicus* and *S. rouxii*. Examples of *Kluyveromyces* sp. are *K. fragilis* and *K. lactis*. Examples of *Hansenula* (*Pichia*) sp. are *H. polymorpha* (now *Pichia angusta*), *H. anomala* (now *P. anomala*) and *P. pastoris*. *Y. lipolytica* is an example of a suitable *Yarrowia*
 20 species.

Methods for the transformation of *S. cerevisiae* are taught generally in EP 251 744, EP 258 067 and WO 90/01063, all of which are incorporated herein by reference. Suitable promoters for *S. cerevisiae* include those associated with
 25 the *PGK1* gene, *GAL1* or *GAL10* genes, *CYC1*, *PHO5*, *TRP1*, *ADH1*, *ADH2*, the genes for glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, triose phosphate isomerase, phosphoglucose isomerase, glucokinase, α -mating factor pheromone, a-mating factor pheromone, the *PRB1* promoter, the *GPD1* promoter, and hybrid
 30 promoters involving hybrids of parts of 5' regulatory regions with parts of 5'

regulatory regions of other promoters or with upstream activation sites (eg the promoter of EP-A-258 067).

Convenient regulatable promoters for use in *Schizosaccharomyces pombe* are
5 the thiamine-repressible promoter from the *nmt* gene as described by Maundrell (1990) and the glucose-repressible *fbp1* gene promoter as described by Hoffman & Winston (1990).

Methods of transforming *Pichia* for expression of foreign genes are taught in,
10 for example, Cregg *et al* (1993), and various Phillips patents (eg US 4 857 467, incorporated herein by reference), and *Pichia* expression kits are commercially available from Invitrogen BV, Leek, Netherlands, and Invitrogen Corp., San Diego, California. Suitable promoters include *AOX1* and *AOX2*.

15 The Gellissen *et al* (1992) paper mentioned above and Gleeson *et al* (1986) *J. Gen. Microbiol.* **132**, 3459-3465 include information on *Hansenula* vectors and transformation, suitable promoters being *MOX1* and *FMD1*; whilst EP 361 991, Fleer *et al* (1991) and other publications from Rhône-Poulenc Rorer teach how to express foreign proteins in *Kluyveromyces* spp., a suitable promoter being
20 *PGK1*.

The transcription termination signal is preferably the 3' flanking sequence of a eukaryotic gene which contains proper signals for transcription termination and polyadenylation. Suitable 3' flanking sequences may, for example, be
25 those of the gene naturally linked to the expression control sequence used, ie may correspond to the promoter. Alternatively, they may be different in which case the termination signal of the *S. cerevisiae ADH1* gene is preferred.

The albumin is initially expressed with a secretion leader sequence, which may
30 be any leader effective in the yeast chosen. Leaders useful in *S. cerevisiae*

include that from the mating factor α polypeptide (MF α -1) and the hybrid leaders of EP-A-387 319. Such leaders (or signals) are cleaved by the yeast before the mature albumin is released into the surrounding medium. When the yeast strain lacks Kex2p activity (or equivalent) as well as being *yap3*, it may

5 be advantageous to choose a secretion leader which need not be cleaved from the albumin by Kex2p. Such leaders include those of *S. cerevisiae* invertase (*SUC2*) disclosed in JP 62-096086 (granted as 91/036516), acid phosphatase (*PHO5*), the pre-sequence of MF α -1, β -glucanase (*BGL2*) and killer toxin; *S. diastolicus* glucoamylase II; *S. carlsbergensis* α -galactosidase (*MEL1*); *K. lactis*

10 killer toxin; and *Candida* glucoamylase.

Various non-limiting embodiments of the invention will now be described by way of example and with reference to the accompanying drawings in which:

15 Figure 1 is a general scheme for the construction of mutated rHA expression plasmids, in which HA is a human albumin coding sequence, L is a sequence encoding a secretion leader, P is the PRB1 promoter, T is the ADH1 terminator, amp is an ampicillin resistance gene and LEU2 is the leucine selectable marker;

20

Figure 2 is a drawing representing a Western blot analysis of mutant rHA secreted by *S. cerevisiae*, in which Track A represents the culture supernatant from DB1 cir^o pAYE316 (normal rHA), Track B represents the culture supernatant from DB1 cir⁺ pAYE464 (alteration 1), and Track C represents the

25 culture supernatant from DB1 cir⁺ pAYE468 (alteration 3);

Figure 3 is a scheme of the construction of pAYE515;

Figure 4 is a comparison of rHA fragment production by wild-type and

30 protease-disrupted strains, presented as a drawing of an anti-HSA Western blot

of culture supernatant from shake flask cultures separated by non-reducing 10% SDS/PAGE, in which Track A corresponds to DB1 cir^o pAYE316, Track B corresponds to DXY10 cir^o pAYE316 (*yap3* strain), and Track C corresponds to ABB50 cir^o pAYE316 (*yap3*, *kex2* strain);

5

Figure 5 is similar to Figure 4 but shows Coomassie Brilliant Blue stained 12.5% SDS Phastgel (Pharmacia) of culture supernatants from fed batch fermentations, namely Track D for the HSA standard, Track E for DB1 cir^o pAYE316, Track F for DB1 $\Delta kex2$ cir^o pAYE522, and Track G for DXY10
10 cir^o pAYE522; and

Figure 6 is a scheme for the construction of pAYE519.

Detailed description of the invention

15

All standard recombinant DNA procedures are as described in Sambrook *et al* (1989) unless otherwise stated. The DNA sequences encoding HSA are derived from the cDNA disclosed in EP 201 239.

20 **Example 1: Modification of the HSA cDNA.**

In order to investigate the role of endoproteases in the generation of rHA fragment, the HSA cDNA (SEQ1 (which includes a sequence encoding the artificial secretion leader sequence of WO 90/01063)) was modified by site-
25 directed mutagenesis. Three separate changes were made to the HSA sequence (SEQ2). The first, using the mutagenic primer FOG1, changed the Arg⁴¹⁰ codon only, replacing it with an Ala codon, leaving intact the dibasic site, Lys⁴¹³Lys⁴¹⁴. The second change, using primer FOG2, changed the residues 407-409, including the C-terminal residues of fragment, from LeuLeuVal to
30 AlaValAla. The third change, using the primer FOG3, altered residues 410-

414 from ArgTyrThrLysLys (SEQ3) to AlaTyrThrGlnGln (SEQ4). The oligonucleotides encoded not only the amino acid changes, but also conservative base changes that create either a *PvuII* or an *SpeI* restriction site in the mutants to facilitate detection of the changed sequences.

5

Single-stranded DNA of an M13mp19 clone, mp19.7 (EP 201 239; Figure 2), containing the HSA cDNA was used as the template for the mutagenesis reactions using the *In Vitro* Mutagenesis System, Version 2 (Amersham International plc) according to the manufacturer's instructions. Individual
10 plaques were selected and sequenced to confirm the presence of the mutations. Double stranded RF DNA was then made from clones with the expected changes and the DNA bearing the mutation was excised on an *XbaI/SacI* fragment (Figure 1). This was used to replace the corresponding wild-type fragment of pAYE309 (EP 431 880; Figure 2). The presence of the mutated
15 *XbaI/SacI* fragment within the plasmid was checked by digesting with *PvuII* or *SpeI* as appropriate. These *HindIII* fragments were excised and inserted into the expression vector pAYE219 (Figure 1) to generate the plasmids pAYE464 (alteration 1, R410A), pAYE470 (alteration 2, L407A, L408V, V409A) and pAYE468 (alteration 3, R410A, K413Q, K414Q). These expression plasmids
20 comprise the *S. cerevisiae PRB1* promoter (WO 91/02057) driving expression of the HSA/MF α 1 leader sequence (WO 90/01063) fused in-frame with the mutated HA coding sequence which is followed by the *ADHI* transcription terminator. The plasmids also contain part of the 2 μ m plasmid to provide replication functions and the *LEU2* gene for selection of transformants.

25

pAYE464, pAYE470 and pAYE468 were introduced into *S. cerevisiae* DB1 cir⁺ (*a*, *leu2*; Sleep *et al*, 1990) by transformation and individual transformants were grown for 3 days at 30°C in 10ml YEPS (1% w/v yeast extract, 2% w/v peptone, 2% w/v sucrose) and then the supernatants were examined by anti-HSA
30 Western blot for the presence of the rHA fragment. The Western blots clearly

showed that fragment was still produced by the strains harbouring pAYE464, although the level was reduced slightly compared to the control expressing wild-type rHA. The mutations in the plasmid pAYE470 appeared to have no effect on the generation of fragment. However, DB1 cir⁺ pAYE468 showed
 5 a novel pattern of HSA-related bands, with little or no fragment.

One example of each of DB1 cir⁺ pAYE464 and DB1 cir⁺ pAYE468 were grown to high cell density by fed batch culture in minimal medium in a fermenter (Collins, 1990). Briefly, a fermenter of 10L working volume was
 10 filled to 5L with an initial batch medium containing 50 mL/L of a concentrated salts mixture (Table 1), 10 mL/L of a trace elements solution (Table 2), 50 mL/L of a vitamins mixture (Table 3) and 20 g/L sucrose. An equal volume of feed medium containing 100 mL/L of the salts mixture, 20 mL/L of the trace elements mixture, 100 mL/L of vitamins solution and 500 g/L sucrose
 15 was held in a separate reservoir connected to the fermenter by a metering pump. The pH was maintained at 5.7 ± 0.2 by the automatic addition of ammonium hydroxide or sulphuric acid, and the temperature was maintained at 30°C. The stirrer speed was adjusted to give a dissolved oxygen tension of >20% air saturation at 1 v/v/min air flow rate.

20

Table 1. Salts Mixture

Chemical	Concentration (g/L)
KH ₂ PO ₄	114.0
MgSO ₄	12.0
CaCl ₂ .6H ₂ O	3.0
Na ₂ EDTA	2.0

25

Table 2. Trace Elements Solution

Chemical	Concentration (g/L)
ZnSO ₄ ·7H ₂ O	3.0
FeSO ₄ ·7H ₂ O	10.0
MnSO ₄ ·4H ₂ O	3.2
CuSO ₄ ·5H ₂ O	0.079
H ₃ BO ₃	1.5
KI	0.2
Na ₂ MoO ₄ ·2H ₂ O	0.5
CoCl ₂ ·6H ₂ O	0.56
H ₃ PO ₄	75mL/L

Table 3. Vitamins Solution

Chemical	Concentration (g/L)
Ca pantothenate	1.6
Nicotinic acid	1.2
m inositol	12.8
Thiamine HCl	0.32
Pyridoxine HCl	0.8
Biotin	0.008

The fermenter was inoculated with 100 mL of an overnight culture of *S. cerevisiae* grown in buffered minimal medium (Yeast nitrogen base [without amino acids, without ammonium sulphate, Difco] 1.7 g/L, (NH₄)₂SO₄ 5 g/L, citric acid monohydrate 6.09 g/L, Na₂HPO₄ 20.16 g/L, sucrose 20 g/L, pH6.5). The initial batch fermentation proceeded until the carbon source had been consumed, at which point the metering pump was switched on and the addition of feed was computer controlled (the micro MFCS system, B. Braun,

Melsungen, Germany) using an algorithm based on that developed by Wang *et al* (1979). A mass spectrometer was used in conjunction with the computer control system to monitor the off gases from the fermentation and to control the addition of feed to maintain a set growth rate (eg 0.1 h⁻¹). Maximum conversion of carbon substrate into biomass is achieved by maintaining the respiratory coefficient below 1.2 (Collins, 1990) and, by this means, cell densities of approximately 100 g/L cell dry weight can be achieved. The culture supernatants were compared with those of a wild-type rHA producer by Coomassie-stained SDS/PAGE and by Western blot. These indicated (Figure 2) that, whilst elimination of the monobasic Arg⁴¹⁰ (pAYE464) did reduce the level of the fragment by a useful amount, removal of both potential protease sites (pAYE468) almost abolished the 45kDa fragment.

The above data suggested that the generation of rHA fragment might be due to endoproteolytic attack, though the absence of an effect of removal of the potential Kex2p site Lys⁴¹³Lys⁴¹⁴ (Sleep *et al*, 1990, and confirmed by other studies not noted here) unless combined with elimination of Arg⁴¹⁰, had suggested a complex etiology. The reduction in the amount of fragment with the mutated rHA could in principle be due to an effect of the changes on the kinetics of folding of the molecule and not due to the removal of protease cleavage sites.

Example 2: Disruption of the YAP3 gene.

The YAP3 gene encoding yeast aspartyl protease 3 was mutated by the process of gene disruption (Rothstein 1983) which effectively deleted part of the YAP3 coding sequence, thereby preventing the production of active Yap3p.

Four oligonucleotides suitable for PCR amplification of the 5' and 3' ends of the YAP3 gene (Egel-Mitani *et al*, 1990) were synthesised using an Applied

Biosystems 380B Oligonucleotide Synthesiser. To assist the reader, we include as SEQ15 the sequence of the *YAP3* gene, of which 541-2250 is the coding sequence.

5 **5' end**

YAP3A: 5'-CGTCAGACCTTGCATGCAGCCAAGACACCCTCACATAGC-3' (SEQ5)

YAP3B: 5'-CCGTTACGTTCTGTGGTGGCATGCCCACTTCCAAGTCCACCG-3' (SEQ6)

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3' end

YAP3C: 5'-GCGTCTCATAGTGGAAAAGCTTCTAAATACGACAACTTCCCC-3' (SEQ7)

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YAP3D: 5'-CCCAAATGGTACCTGTGTCATCACTCGTTGGGATAATACC-3' (SEQ8)

PCR reactions were carried out to amplify individually the 5' and 3' ends of the *YAP3* gene from *S. cerevisiae* genomic DNA (Clontech Laboratories, Inc). Conditions were as follows: 2.5µg/ml genomic DNA, 5µg/ml of each primer, 20 denature at 94°C 61 seconds, anneal at 37°C 121 secs, extend at 72°C 181 secs for 40 cycles, followed by a 4°C soak, using a Perkin-Elmer-Cetus Thermal Cycler and a Perkin-Elmer-Cetus PCR kit according to the manufacturer's recommendations. Products were analysed by gel electrophoresis and were found to be of the expected size. The 5' fragment 25 was digested with *SphI* and cloned into the *SphI* site of pUC19HX (pUC19 lacking a *HindIII* site) to give pAYE511 (Figure 3), in which the orientation is such that *YAP3* would be transcribed towards the *KpnI* site of the pUC19HX polylinker. The 3' *YAP3* fragment was digested with *HindIII* and *Asp718* (an isoschizomer of *KpnI*) and ligated into pUC19 digested with *HindIII/Asp718* to 30 give pAYE512. Plasmid DNA sequencing was carried out on the inserts to confirm that the desired sequences had been cloned. The *HindIII/Asp718* fragment of pAYE512 was then subcloned into the corresponding sites of pAYE511 to give pAYE513 (Fig 3), in which the 5' and 3' regions of *YAP3*

are correctly orientated with a unique *Hind*III site between them. The *URA3* gene was isolated from YEp24 (Botstein *et al*, 1979) as a *Hind*III fragment and then inserted into this site to give pAYE515 (Fig 3), with *URA3* flanked by the 5' and 3' regions of *YAP3*, and transcribed in the opposite direction to *YAP3*.

5

A *ura3* derivative of strain DB1 *cir*^o pAYE316 (Sleep *et al*, 1991) was obtained by random chemical mutagenesis and selection for resistance to 5-fluoro-orotic acid (Boeke *et al*, 1987). The strain was grown overnight in 100 mL buffered minimal medium and the cells were collected by centrifugation and then washed once with sterile water. The cells were then resuspended in 10 mL sterile water and 2 mL aliquots were placed in separate 15 mL Falcon tubes. A 5 mg/mL solution of N-methyl-N'-nitro-N-nitrosoguanidine (NTG) was then added to the tubes as follows: 0 μ L, 20 μ L, 40 μ L, 80 μ L or 160 μ L. The cells were then incubated at 30°C for 30 min and then centrifuged and washed three times with sterile water. Finally, the cells were resuspended in 1 mL YEP (1% w/v yeast extract, 2% w/v Bacto peptone) and stored at 4°C. The percentage of cells that survived the mutagenic treatment was determined by spreading dilutions of the samples on YEP plates containing 2% w/v sucrose and incubating at 30°C for 3 days. Cells from the treatment which gave approximately 50% survival were grown on YEP plates containing 2% w/v sucrose and then replica-plated onto YNB minimal medium containing 2% w/v sucrose and supplemented with 5-fluoro-orotic acid (1 mg/mL) and uracil (50 μ g/mL). Colonies able to grow on this medium were purified, tested to verify that they were unable to grow in the absence of uracil supplementation and that this defect could be corrected by introduction of the *URA3* gene by transformation. One such strain, DBU3 *cir*^o pAYE316, was transformed with the *Sph*I/*Asp*718 *YAP3-URA3-YAP3* fragment of pAYE515 with selection for Ura⁺ colonies. A Southern blot of digested genomic DNA of a number of transformants was probed with the 5' and 3' ends of the *YAP3* gene and confirmed the disruption of the *YAP3* gene. An anti-HSA Western blot of

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YEPS shake-flask supernatants of two transformants indicated that disruption of *YAP3* markedly reduced rHA fragment levels.

One *yap3* derivative of DBU3 cir^o pAYE316, designated DXY10 cir^o pAYE316, was grown several times by fed-batch fermentation in minimal medium to high cell dry weight. When supernatants were examined by Coomassie-stained PAGE and anti-HSA Western blot (Figs 4 and 5), the reduction in the level of rHA 45kDa fragment was clearly apparent; estimates of the amount of the degradation product vary from $\frac{1}{3}$ to $\frac{1}{5}$ of the levels seen with the *YAP3* parent. The amount of rHA produced was not adversely affected by the *yap3* mutation, indeed DXY10 cir^o pAYE316 was found to produce 30-50% more rHA than the *YAP3* equivalent, DB1 cir^o pAYE316. Despite the fact that cleavage of the leader sequence from the HA sequence is C-terminal to a pair of basic residues, the rHA was found to have the correct N-terminus.

The fermentation broth was centrifuged to remove the cells and then subject to affinity chromatographic purification as follows. The culture supernatant was passed through a Cibacron Blue F3GA Sepharose column (Pharmacia) which was then washed with 0.1M phosphate glycine buffer, pH8.0. The rHA was then eluted from the column with 2M NaCl, 0.1M phosphate glycine, pH8.0, at which point it was >95% pure. It may be purified further by techniques known in the art.

The albumin may alternatively be purified from the culture medium by any of the variety of known techniques for purifying albumin from serum or fermentation culture medium, for example those disclosed in WO 92/04367, Maurel *et al* (1989), Curling (1980) and EP 524 681.

Example 3: Disruption of the *KEX2* gene in a *yap3* strain.

To construct a strain lacking both Yap3p and Kex2p activity, a *lys2* derivative of yeast strain DXY10 *cir*^o (pAYE316) was obtained by random chemical mutagenesis and selection for resistance to α -amino adipate (Barnes and Thorner, 1985). Cells were mutagenised as in Example 2 and then plated on YNB minimal medium containing 2% w/v sucrose and supplemented with 2 mg/mL DL- α -amino adipate as the sole nitrogen source and 30 μ g/mL lysine. Colonies able to grow on this medium were purified and tested to verify that they were unable to grow in the absence of lysine supplementation and that this defect could be corrected by the introduction of the *LYS2* gene by transformation. This strain was then mutated by the process of gene disruption which effectively disrupted part of the *KEX2* coding sequence, thereby preventing production of active Kex2p. To assist the reader, the sequence of the *KEX2* gene is reproduced herein as SEQ14, of which 1329-3773 is the coding sequence.

Four oligonucleotides suitable for PCR amplification of the 5' and 3' ends of the *KEX2* gene (Fuller *et al*, 1989) were synthesised using an Applied Biosystems 380B Oligonucleotide Synthesiser.

5' end

KEX2A: 5'-CCATCTGGATCCAATGGTGCTTTGGCCAAATAAATAGTTTCAGC-3' (SEQ9)
 25 KEX2B: 5'-GCTTCTTTTACCGGTAACAAGCTTGAGTCCATTGG-3' (SEQ10)

3' end

KEX2C: 5'-GGTAAGGTTTAGTCGACCTATTTTTTGTGTTTGTCTGC-3' (SEQ11)
 30 KEX2D: 5'-GGAAACGTATGAATTCGATATCATTGATACAGACTCTGAGTACG-3' (SEQ12)

PCR reactions were carried out to amplify individually the 5' and 3' ends of the *KEX2* gene from *S. cerevisiae* genomic DNA (Clontech Laboratories Inc). Conditions were as follows: 2.5 µg/ml genomic DNA, 5 µg/ml of each primer, denature 94°C 61s, anneal 37°C 121s, extend 72°C 181s for 40 cycles, followed by a 4°C soak, using a Perkin-Elmer-Cetus Thermal Cycler and a Perkin-Elmer-Cetus PCR kit according to the manufacturer's recommendations. Products were analysed by gel electrophoresis and were found to be of the expected size (0.9 kb for the 5' product and 0.62 kb for the 3' product). The 5' product was digested with *Bam*HI and *Hind*III and the 3' product was digested with *Hind*III and *Sal*I and then the two fragments were together cloned into pUC19HX digested with *Bam*HI and *Sal*I. A 4.8 kb *Hind*III fragment comprising the *S. cerevisiae* *LYS2* gene (Barnes & Thorner, 1985) was then inserted into the resulting plasmid at *Hind*III (ie between the two *KEX2* fragments) to form pAYE519 (Fig 6).

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The *lys2* derivative of DXY10 cir° (pAYE316), *lys2-16*, was transformed with the 6.0 kb *KEX2-LYS2-KEX2* fragment of pAYE519, selecting for Lys⁺ colonies. A Southern blot of digested genomic DNA of a number of transformants was probed with the 5' and 3' ends of the *KEX2* gene and confirmed the disruption of the *KEX2* gene. An anti-HSA Western blot of YEPS shake-flask culture supernatants of these transformants indicated that disruption of *KEX2* in a *yap3* strain reduced the level of rHA fragment still further, despite the lack of an effect of disruption of *KEX2* alone in Example 4 below. Analysis of the rHA produced by one such strain, ABB50, indicated that the leader sequence was incorrectly processed, leading to an abnormal N-terminus.

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The strain ABB50 (pAYE316) was cured of its plasmid (Sleep *et al*, 1991) and transformed with a similar plasmid, pAYE522, in which the hybrid leader sequence was replaced by the *S. cerevisiae* invertase (*SUC2*) leader sequence

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5 Invertase leader HSA

Example 4: Disruption of the *KEX2* gene alone (Comparative Example).

Example 5: Identification of equivalent protease in *Pichia*.

25 As noted above, non-*Saccharomyces* yeast similarly produce the undesirable fragment of rHA and therefore have the Yap3p proteolytic activity. We have confirmed this by performing Southern hybridisations of *Pichia angusta* DNA, using the *S. cerevisiae* *YAP3* gene as a probe. A specific DNA fragment was identified, showing that, not only is the Yap3p proteolytic activity present in
30 *P. angusta*, but a specific homologue of the *YAP3* gene is present also.

The method of Southern hybridization used for detection of the *YAP3* homologue can be adapted to clone the gene sequence from a genomic DNA library of *Pichia* DNA using standard procedures (Sambrook *et al*, 1989). Disruption of the *YAP3* homologue in *Pichia sp.* can be achieved using similar
5 techniques to those used above for *Saccharomyces* (Cregg and Madden, 1987).

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

- (A) NAME: Delta Biotechnology Limited
- (B) STREET: Castle Court, Castle Boulevard
- (C) CITY: Nottingham
- (D) STATE: Nottinghamshire
- (E) COUNTRY: United Kingdom
- (F) POSTAL CODE (ZIP): NG7 1FD

(ii) TITLE OF INVENTION: Yeast strains and modified albumins

(iii) NUMBER OF SEQUENCES: 15

(iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1830 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Homo sapiens

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 73..1827

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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320				325				330								
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GTC Val	GAT Asp	GAA Glu 495	ACA Thr	TAC Tyr	GTT Val	CCC Pro	AAA Lys 500	GAG Glu	TTT Phe	AAT Asn	GCT Ala	GAA Glu 505	ACA Thr	TTC Phe	ACC Thr	1596
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AAG Lys	TGC Cys	TGC Cys	AAG Lys 560	GCT Ala	GAC Asp	GAT Asp	AAG Lys	GAG Glu 565	ACC Thr	TGC Cys	TTT Phe	GCC Ala	GAG Glu 570	GAG Glu	GGT Gly	1788
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(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 585 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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          85           90           95
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Pro Arg Leu Val Arg Pro Glu Val Asp Val Met Cys Thr Ala Phe His
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Asp Asn Glu Glu Thr Phe Leu Lys Lys Tyr Leu Tyr Glu Ile Ala Arg
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Arg His Pro Tyr Phe Tyr Ala Pro Glu Leu Leu Phe Phe Ala Lys Arg
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Tyr Lys Ala Ala Phe Thr Glu Cys Cys Gln Ala Ala Asp Lys Ala Ala
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Cys Leu Leu Pro Lys Leu Asp Glu Leu Arg Asp Glu Gly Lys Ala Ser
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Ser Ala Lys Gln Arg Leu Lys Cys Ala Ser Leu Gln Lys Phe Gly Glu
          195          200          205
Arg Ala Phe Lys Ala Trp Ala Val Ala Arg Leu Ser Gln Arg Phe Pro
          210          215          220
Lys Ala Glu Phe Ala Glu Val Ser Lys Leu Val Thr Asp Leu Thr Lys
          225          230          235          240
Val His Thr Glu Cys Cys His Gly Asp Leu Leu Glu Cys Ala Asp Asp
          245          250          255
Arg Ala Asp Leu Ala Lys Tyr Ile Cys Glu Asn Gln Asp Ser Ile Ser
          260          265          270
Ser Lys Leu Lys Glu Cys Cys Glu Lys Pro Leu Leu Glu Lys Ser His
          275          280          285
Cys Ile Ala Glu Val Glu Asn Asp Glu Met Pro Ala Asp Leu Pro Ser

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Tyr Val Pro Lys Glu Phe Asn Ala Glu Thr Phe Thr Phe His Ala Asp 500 505 510		
Ile Cys Thr Leu Ser Glu Lys Glu Arg Gln Ile Lys Lys Gln Thr Ala 515 520 525		
Leu Val Glu Leu Val Lys His Lys Pro Lys Ala Thr Lys Glu Gln Leu 530 535 540		
Lys Ala Val Met Asp Asp Phe Ala Ala Phe Val Glu Lys Cys Cys Lys 545 550 555 560		
Ala Asp Asp Lys Glu Thr Cys Phe Ala Glu Glu Gly Lys Lys Leu Val 565 570 575		
Ala Ala Ser Gln Ala Ala Leu Gly Leu 580 585		

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Arg Tyr Thr Lys Lys
1 5

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 5 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Ala Tyr Thr Gln Gln
1 5

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 39 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

CGTCAGACCT TGCATGCAGC CAAGACACCC TCACATAGC

39

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 42 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

CCGTTACGTT CTGTGGTGGC ATGCCCACTT CCAAGTCCAC CG

42

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 42 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

GCGTCTCATA GTGGAAAAGC TTCTAAATAC GACAACTTCC CC

42

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 41 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

CCCAAAATGG TACCTGTGTC ATCACTCGTT GGGATAATAC C

41

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 44 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

CCATCTGGAT CCAATGGTGC TTTGGCCAAA TAAATAGTTT CAGC

44

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 35 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

GCTTCTTTTA CCGTAACAA GCTTGAGTCC ATTGG

35

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 37 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

GGTAAGGTTT AGTCGACCTA TTTTGTGTTT TGTCTGC

37

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 44 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

GGAAACGTAT GAATTCGATA TCATTGATAC AGACTCTGAG TACG

44

(2) INFORMATION FOR SEQ ID NO: 13:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 24 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(v) FRAGMENT TYPE: N-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

Met Leu Leu Gln Ala Phe Leu Phe Leu Leu Ala Gly Phe Ala Ala Lys
 1 5 10 15

Ile Ser Ala Asp Ala His Lys Ser
 20

(2) INFORMATION FOR SEQ ID NO: 14:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 4106 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: *Saccharomyces cerevisiae*

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

GAATTCTCTG TTGACTACTA AACTGAGAGA ATTTGCCGAG ACTCTAAGAA CAGCTTTGAA	60
AGAGCGTTCT GCCGATGATT CCATAATTGT CACTCTGAGA GAGCAAATGC AAAGAGAAAT	120
CTTCAGGTTG ATGTCGTTGT TCATGGACAT ACCTCCAGTG CAACCAAACG AGCAATTCAC	180
TTGGGAATAC GTTGACAAAG ACAAGAAAAT CCACACTATC AAATCGACTC CGTTAGAATT	240
TGCCTCCAAA TACGCAAAT TGGACCCTTC CAGCCAGTC TCATTGATCA ATGATCCAAG	300
ACACCATATG GTAAATTAAT TAAGATCGAT CGTTTAGGAA ACGTCCTTGG CGGAGATGCC	360
GTGATTTACT TAAATGTTGA CAATGAAACA CTATCTAAAT TGGTTGTTAA GAGATTACAA	420
AATAACAAAG CTGTCTTTTT TGGATCTCAC ACTCCAAAGT TCATGGACAA GAAAAGTGGT	480
GTCATGGATA TTGAATTGTG GAACTATCCT GCCATGGCTA TAATTTACCT CAGCAAAGG	540
CATCCGGTAT TAGATACCAT GAAAGTTTGA TGACTCATGC TATGTTGGAT CACTGGCTGC	600
CACGTCGATG AAACGTCTAA ATTACCACTT CGCTACCGTC TGAAAATTCC TGGGGTAAAG	660

ACTCCGGTAA AGACGGATTA TACGTGATGA CTCAAAAGTA CTTCGAGGAG TACTGCTTTC	720
AAATTGTGGT CGATATCAAT GAATTGCCAA AAGAGCTGGC TTCAAAATTC ACCTCAGGTA	780
AGGAAGAGCC GATTGTCTTG CCCATCTGGA CCCAATGGTG CTTTGGCCAA ATAAATAGTT	840
TCAGCAGCTC TGATGTAGAT ACACGTATCT CGACATGTTT TATTTTACT ATACATACAT	900
AAAAGAAATA AAAAATGATA ACGTGTATAT TATTATTCAT ATAATCAATG AGGGTCATTT	960
TCTGAAACGC AAAAACCGGT AAATGGAAAA AAAATAAAGA TAGAAAAAGA AAACAAACAA	1020
AGGAAAGGTT AGCATATTAA ATAAGTGGC TGATACTTCA ACAGCATCGC TGAAGAGAAC	1080
AGTATTGAAA CCGAAACATT TTCTAAAGGC AAACAAGGTA CTCCATATTT GCTGGACGTG	1140
TTCTTTCTCT CGTTTCATAT GCATAATTCT GTCATAAGCC TGTTCTTTT CCTGGCTTAA	1200
ACATCCCGTT TTGTAAAAGA GAAATCTATT CCACATATTT CATTCAATTCG GCTACCATAC	1260
TAAGGATAAA CTAATCCCGT TGTTTTTTGG CCTCGTCACA TAATTATAAA CTACTAACCC	1320
ATTATCAGAT GAAAGTGAGG AAATATATTA CTTTATGCTT TTGGTGGGCC TTTTCAACAT	1380
CCGCTCTTGT ATCATCACAA CAAATTCAT TGAAGGACCA TACGTCACGA CAGTATTTTG	1440
CTGTAGAAAAG CAATGAAACA TTATCCCGCT TGGAGGAAAT GCATCCAAAT TGGAAATATG	1500
AACATGATGT TCGAGGGCTA CCAAACCATT ATGTTTTTTC AAAAGAGTTG CTAAAATTGG	1560
GCAAAAGATC ATCATTAGAA GAGTTACAGG GGGATAACAA CGACCACATA TTATCTGTCC	1620
ATGATTTATT CCCGCGTAAC GACCTATTTA AGAGACTACC GGTGCCTGCT CCACCAATGG	1680
ACTCAAGCTT GTTACCGGTA AAAGAAGCTG AGGATAAACT CAGCATAAAT GATCCGCTTT	1740
TTGAGAGGCA GTGGCACTTG GTCAATCCAA GTTTTCTTG CAGTGATATA AATGTTCTTG	1800
ATCTGTGGTA CAATAATATT ACAGGCGCAG GGGTCGTGGC TGCCATTGTT GATGATGGCC	1860
TTGACTACGA AAATGAAGAC TTGAAGGATA ATTTTTCGCG TGAAGGTTCT TGGGATTTCA	1920
ACGACAATAC CAATTTACCT AAACCAAGAT TATCTGATGA CTACCATGGT ACGAGATGTG	1980
CAGGTGAAAT AGCTGCCAAA AAAGGTAACA ATTTTTCGCG TGTCGGGGTA GGTTACAACG	2040
CTAAAATCTC AGGCATAAGA ATCTTATCCG GTGATATCAC TACGGAAGAT GAAGCTGCGT	2100
CCTTGATTTA TGGTCTAGAC GTAAACGATA TATATTCATG CTCATGGGGT CCCGCTGATG	2160
ACGGAAGACA TTTACAAGGC CCTAGTGACC TGGTGAAAAA GGCTTTAGTA AAAGGTGTTA	2220
CTGAGGGAAG AGATTCCAAA GGAGCGATTT ACGTTTTTGC CAGTGGAAT GGTGGAATC	2280
GTGGTGATAA TTGCAATTAC GACGGCTATA CTAATCCAT ATATTCTATT ACTATTGGGG	2340
CTATTGATCA CAAAGATCTA CATCCTCCTT ATTCCGAAGG TTGTTCCGCC GTCATGGCAG	2400
TCACGTATTC TTCAGGTTCA GGCGAATATA TTCATTGAG TGATATCAAC GGCAGATGCA	2460
GTAATAGCCA CGGTGGAACG TCTGCGGCTG CTCCATTAGC TGCCGGTGTT TACACTTTGT	2520
TACTAGAAGC CAACCCAAAC CTAAGTTGGA GAGACGTACA GTATTTATCA ATCTTGTCTG	2580
CGGTAGGGTT AGAAAAGAAC GCTGACGGAG ATTGGAGAGA TAGCGCCATG GGAAGAAAT	2640

ACTCTCATCG CTATGGCTTT GGTAAATCG ATGCCCATAA GTTAATTGAA ATGTCCAAGA	2700
CCTGGGAGAA TGTTAACGCA CAAACCTGGT TTTACCTGCC AACATTGTAT GTTCCCACT	2760
CCACAACTC CACGGAAGAG ACATTAGAAT CCGTCATAAC CATATCAGAA AAAAGTCTTC	2820
AAGATGCTAA CTTCAAGAGA ATTGAGCACG TCACGGTAAC TGTAATATT GATACAGAAA	2880
TTAGGGGAAC TACGACTGTC GATTTAATAT CACCAGCGGG GATAATTTCA AACCTTGGCG	2940
TTGTAAGACC AAGAGATGTT TCATCAGAGG GATTCAAAGA CTGGACATTC ATGTCTGTAG	3000
CACATTGGGG TGAGAACGGC GTAGGTGATT GGAAATCAA GGTTAAGACA ACAGAAAATG	3060
GACACAGGAT TGAATTCAC AGTTGGAGGC TGAAGCTCTT TGGGGAATCC ATTGATTCAT	3120
CTAAACAGA AACTTTCGTC TTTGGAAACG ATAAAGAGGA GGTGAACCA GCTGCTACAG	3180
AAAGTACCGT ATCACAATAT TCTGCCAGTT CAACTTCTAT TTCCATCAGC GCTACTTCTA	3240
CATCTTCTAT CTCAATTGGT GTGGAAACGT CGGCCATTCC CCAAACGACT ACTGCGAGTA	3300
CCGATCCTGA TTCTGATCCA AACACTCCTA AAAAATTTT CTCTCCTAGG CAAGCCATGC	3360
ATTATTTTTT AACAATATTT TTGATTGGCG CCACATTTTT GGTGTTATAC TTCATGTTTT	3420
TTATGAAATC AAGGAGAAGG ATCAGAAGGT CAAGAGCGGA AACGTATGAA TTCGATATCA	3480
TTGATACAGA CTCTGAGTAC GATTCTACTT TGGACAATGG AACTCCGGA ATTACTGAGC	3540
CCGAAGAGGT TGAGGACTTC GATTTTGATT TGTCCGATGA AGACCATCTT GCAAGTTTGT	3600
CTTCATCAGA AAACGGTGAT GCTGAACATA CAATTGATAG TGTACTAACA AACGAAAATC	3660
CATTTAGTGA CCCTATAAAG CAAAAGTTCC CAAATGACGC CAACGCAGAA TCTGCTTCCA	3720
ATAAATTACA AGAATTACAG CCTGATGTTT CTCCATCTTC CGGACGATCG TGATTCGATA	3780
TGTACAGAAA GCTTCAAATT ACAAATAGC ATTTTTTTCT TATAGATTAT AATACTCTCT	3840
CATACGTATA CGTATATGTG TATATGATAT ATAAACAAAC ATTAATATCC TATTCCTTCC	3900
GTTTGAAATC CCTATGATGT ACTTTGCATT GTTTGCACCC GCGAATAAAA TGAAAATCC	3960
GAACCGATAT ATCAAGCACA TAAAGGGGA GGGTCCAATT AATGCATATT TAAGACCACA	4020
GCTGAATAAC TTAAAACGG CAGACAAAAC AAAAATAGG TCGAATAAAC CTTACCTGCC	4080
TAGAAGGAAT GACAGCAGCT AATAAG	4106

(2) INFORMATION FOR SEQ ID NO: 15:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 2526 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (iii) HYPOTHETICAL: NO
- (iii) ANTI-SENSE: NO
- (vi) ORIGINAL SOURCE:
 - (A) ORGANISM: *Saccharomyces cerevisiae*

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

CCGTTTTCTT TTCGTAAAA AAAACAATAG ACACTATATA TAGACACTTT TTCCTTTCCT	60
TCTTTGCGCG ATTTCAAGAG GAAAAGCATA CTTAAATAAG AATATTCCTA AAACACACGT	120
TCTGACGCGT CAATTAGATC GTCAGACCTT GCATGCAGCC AAGACACCCT CACATAGCAC	180
TGCCTCCTTC CTCCTCTTTT CTGTCACCAC CTCACCTCCC TCGTCCACTC AACTGAGTGG	240
CTTTTCGCTC CTTTTATACT GCGCCATGAG TAGTTTTCGT TTTACTGATG TGTCCGAAAA	300
AATTGAGGTT TCATAAAAAA ATTCGTGGAC TTATTTATGG AGAAACAGGG AAATCCGACT	360
ACTTAAGAAA AGGGTGTCAA AGAGGATTTA CTTTTTTCCT TCTTTTGGCA TTTGTTCTTA	420
TTTCCGCAAT TGGACGGTTA TTAAGAAGAA CGCAATTGGC TTTTCTGTAT ATTAAAATAC	480
ATAGCGTAAT AAAAAGATAA GGTGAACACC AAGCATATAG TATAATATTA CCTACCACAT	540
ATGAAACTGA AAAGTGAAG ATCTGCGGTC CTTTCGTCAC TCTTTGCATC GCAGGTTCTC	600
GGTAAGATAA TACCAGCAGC AAACAAGCGC GACGACGACT CGAATTCCAA GTTCGTCAAG	660
TTGCCCTTTC ATAAGCTTTA CGGGGACTCG CTAGAAAATG TGGGAAGCGA CAAAAACCG	720
GAAGTACGCC TATTGAAGAG GGCTGACGGT TATGAAGAAA TTATAATTAC CAACCAGCAA	780
AGTTTCTATT CGGTGGACTT GGAAGTGGGC ACGCCACCAC AGAACGTAAC GGTCTGGTG	840
GACACAGGCT CCTCTGATCT ATGGATTATG GGCTCGGATA ATCCATACTG TTCTTCGAAC	900
AGTATGGGTA GTAGCCGGAG ACGTGTTATT GACAAACGTG ATGATTTCGT CAGCGGCGGA	960
TCTTTGATTA ATGATATAAA CCCATTTGGC TGGTTGACGG GAACGGGCAG TGCCATTGGC	1020
CCCCTGCTA CGGGCTTAGG AGGCGGTTCA GGTACGGCAA CTCAATCCGT GCCTGCTTCG	1080
GAAGCCACCA TGGACTGTCA ACAATACGGG ACATTTTCCA CTTGCGGCTC TTCTACATTT	1140
AGATCAAACA ACACCTATTT CAGTATTAGC TACGGTGATG GGACTTTTGC CTCCGGTACT	1200
TTTGGTACGG ATGTTTTGGA TTTAAGCGAC TTGAACGTTA CCGGGTTGTC TTTTGCCGTT	1260
GCCAATGAAA CGAATTCTAC TATGGGTGTG TTAGGTATTG GTTTGCCCCA ATTAGAAGTC	1320
ACTTATTCTG GCTCTACTGC GTCTCATAGT GGAAAAGCTT ATAAATACGA CAACTTCCCC	1380
ATTGTATTGA AAAATTCTGG TGCTATCAAA AGCAACACAT ATTCTTTGTA TTTGAACGAC	1440
TCGGACGCTA TGCATGGCAC CATTTTGTTT GGAGCCGTGG ACCACAGTAA ATATACCGGC	1500
ACCTTATACA CAATCCCCAT CGTAAACACT CTGAGTGCTA GTGGATTTAG CTCTCCCAT	1560
CAATTTGATG TCACTATTAA TGGTATCGGT ATTAGTGATT CTGGGAGTAG TAACAAGACC	1620
TTGACTACCA CTAAAATACC TGCTTTGTCG GATTCCGGTA CTACTTTGAC TTATTTACCT	1680
CAAACAGTGG TAAGTATGAT CGCTACTGAA CTAGGTGCGC AATACTCTTC CAGGATAGGG	1740
TATTACGTAT TGGACTGTCC ATCTGATGAT AGTATGGAAA TAGTGTTTCA TTTGGTGGT	1800
TTTCACATCA ATGCACCACT TTCGAGTTTT ATCTTGAGTA CTGGCACTAC ATGTCTTTTA	1860
GGTATTATCC CAACGAGTGA TGACACAGGT ACCATTTTGG GTGATTCATT TTTGACTAAC	1920

GCGTACGTGG	TTTATGATTT	GGAGAATCTT	GAAATATCCA	TGGCACAAGC	TCGCTATAAT	1980
ACCACAAGCG	AAAATATCGA	AATTATCACA	TCCTCTGTTC	CAAGCGCCGT	AAAGGCACCA	2040
GGCTATACAA	ACACTTGGTC	CACAAGTGCA	TCTATTGTTA	CCGGTGGTAA	CATATTTACT	2100
GTAATTCCT	CACAAACTGC	TTCCTTTAGC	GGTAACCTGA	CGACCAGTAC	TGCATCCGCC	2160
ACTTCTACAT	CAAGTAAAAG	AAATGTTGGT	GATCATATAG	TTCCATCTTT	ACCCCTCACA	2220
TTAATTTCTC	TTCTTTTTGC	ATTCATCTGA	AAACCGTTGC	ACAAAGTTTA	GACATTCACA	2280
TCTCCAAGCC	AGTTGGAGTT	TCTGGCGGAA	ATCGTTGCTC	TCGCTTGGGC	AAAGTTTTTT	2340
TTTATTATTA	ATTTTTTATT	GTTACGTTGG	CGGTCTTTAT	TTTTACTTCA	CAATAGTTTA	2400
TCTTACCCAC	TAAGAATAGG	TTACCATTTA	TTCACATTTT	TTTTTCTCAT	TCCTAGTATA	2460
CTATTTACCT	GGGATATGGC	CTATAATCAA	AGGCTTTAAT	ATTCTAATAA	TTCGTTTGGC	2520
ATCTAG						2526

CLAIMS

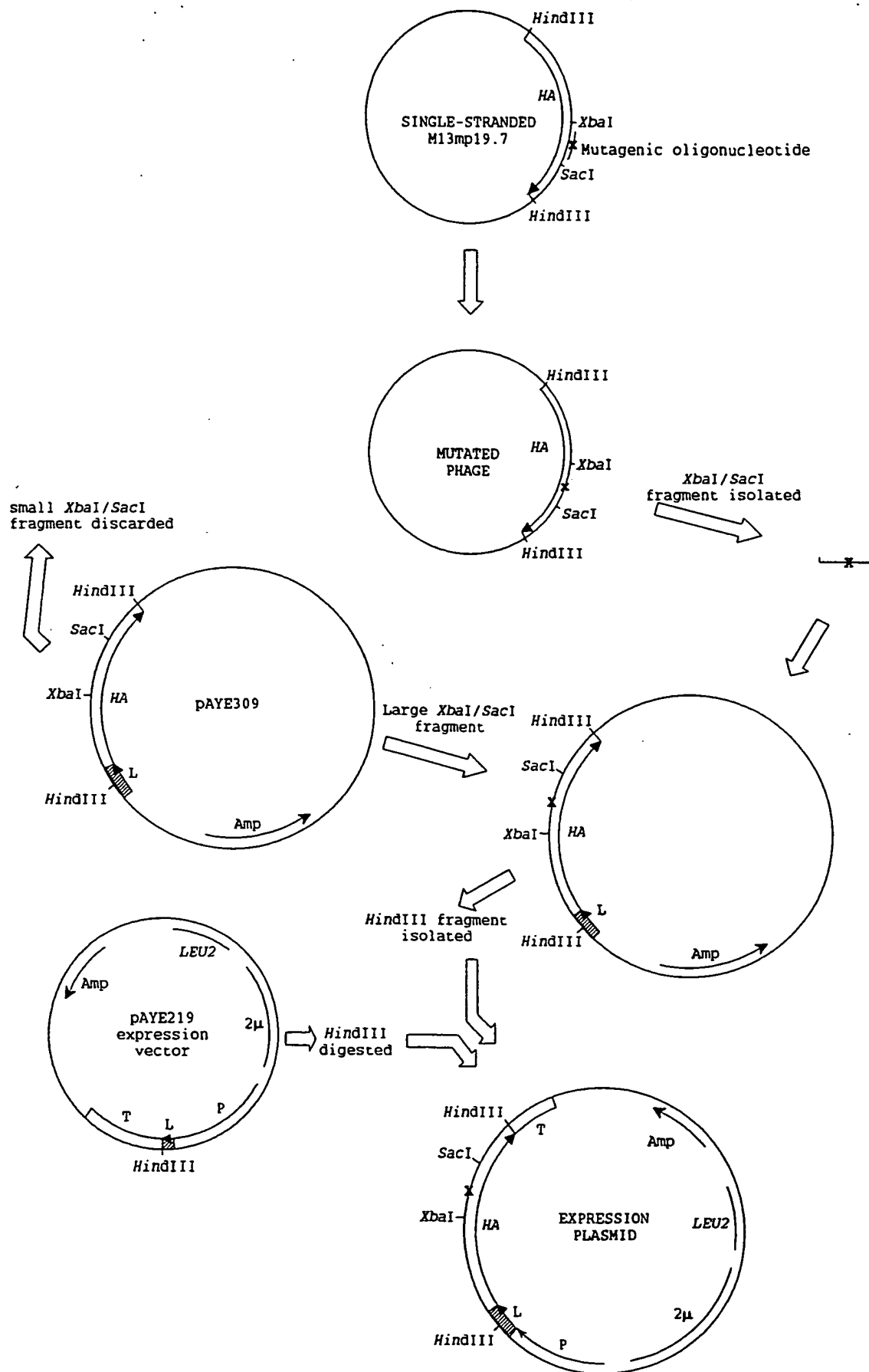
1. A process for preparing albumin by secretion from a yeast genetically modified to produce and secrete the albumin, comprising culturing the yeast in a culture medium such that albumin is secreted into the medium, characterised in that the yeast cells have a reduced level of yeast aspartyl protease 3 proteolytic activity.
2. A process according to Claim 1 wherein the said proteolytic activity is an endoprotease activity specific for monobasic sites and for paired basic amino acids in a polypeptide.
3. A process according to Claim 1 or 2 wherein the yeast is *S. cerevisiae*.
4. A process according to Claim 1, 2 or 3 wherein the yeast lacks a functional *YAP3* gene or homologue thereof.
5. A process according to any one of Claims 1 to 4 wherein the yeast cells additionally have a reduced level of *S. cerevisiae* Kex2p proteolytic activity.
6. A process according to any one of the preceding claims wherein the albumin is a human albumin.
7. A culture of yeast cells containing a polynucleotide sequence encoding an albumin and a second polynucleotide sequence encoding a secretion signal causing albumin expressed from the first polynucleotide sequence to be secreted from the yeast, characterised in that the yeast cells have a reduced level of yeast aspartyl protease 3 proteolytic

activity.

8. A culture according to Claim 7 wherein the albumin is a human albumin.
- 5 9. A culture according to Claim 7 or 8 wherein the yeast is *S. cerevisiae*.
- 10 10. A culture according to any one of Claims 7 to 9 wherein the said signal is cleaved by the yeast prior to release of the albumin from the yeast.
- 15 11. A culture according to any one of Claims 7 to 10 wherein the yeast cells additionally have a reduced level of Kex2p proteolytic activity.
- 20 12. A culture according to Claim 11 wherein the said secretion signal is cleaved from the albumin by a protease other than Kex2p.
- 25 13. A modified albumin having at least 90% sequence identity to a naturally-occurring albumin, which naturally-occurring albumin is susceptible to cleavage with yeast aspartyl protease 3 (Yap3p) when expressed and secreted in yeast, characterised in that the modified albumin is not susceptible to such cleavage.
- 30 14. A modified albumin according to Claim 13 wherein the modified albumin lacks a monobasic amino acid present in the naturally-occurring albumin protein.
15. A modified albumin according to Claim 13 or 14 wherein the said monobasic amino acid is arginine.

16. A modified albumin according to Claim 14 or 15 wherein the modified albumin additionally lacks a pair of basic amino acids present in the naturally-occurring albumin.
- 5 17. A modified albumin according to Claim 16 wherein the said pair of amino acids is Lys, Lys; Lys, Arg; Arg, Lys; or Arg, Arg.
18. A modified albumin according to Claim 13 wherein the naturally-occurring albumin is a human albumin and the modified protein lacks
10 Arg⁴¹⁰; and, optionally, residues 413 and 414 do not each consist of lysine or arginine.
19. A modified albumin according to Claim 18 which is a human albumin having the amino acid changes R410A, K413Q, K414Q.
15
20. A polynucleotide encoding a modified albumin according to any one of Claims 13 to 19.
21. A yeast containing a polynucleotide according to Claim 20,
20 transcription signals such that the modified albumin is expressed in the yeast, and a further polynucleotide adjacent the said polynucleotide such that the modified albumin is secreted from the yeast.

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Figure 1.



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Figure 2

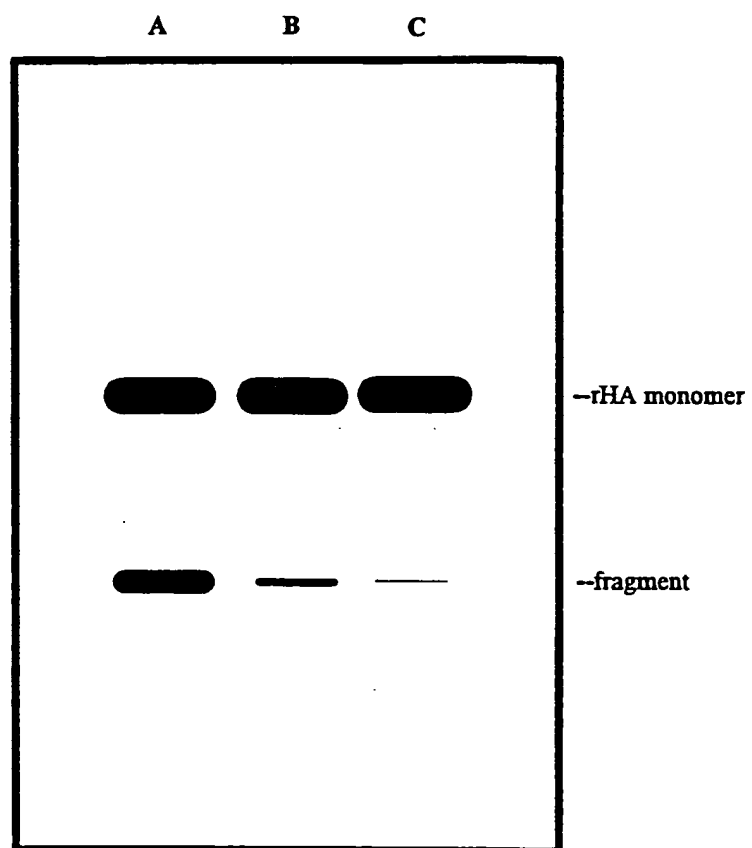


Figure 3

5' and 3' regions of YAP3 obtained by PCR:

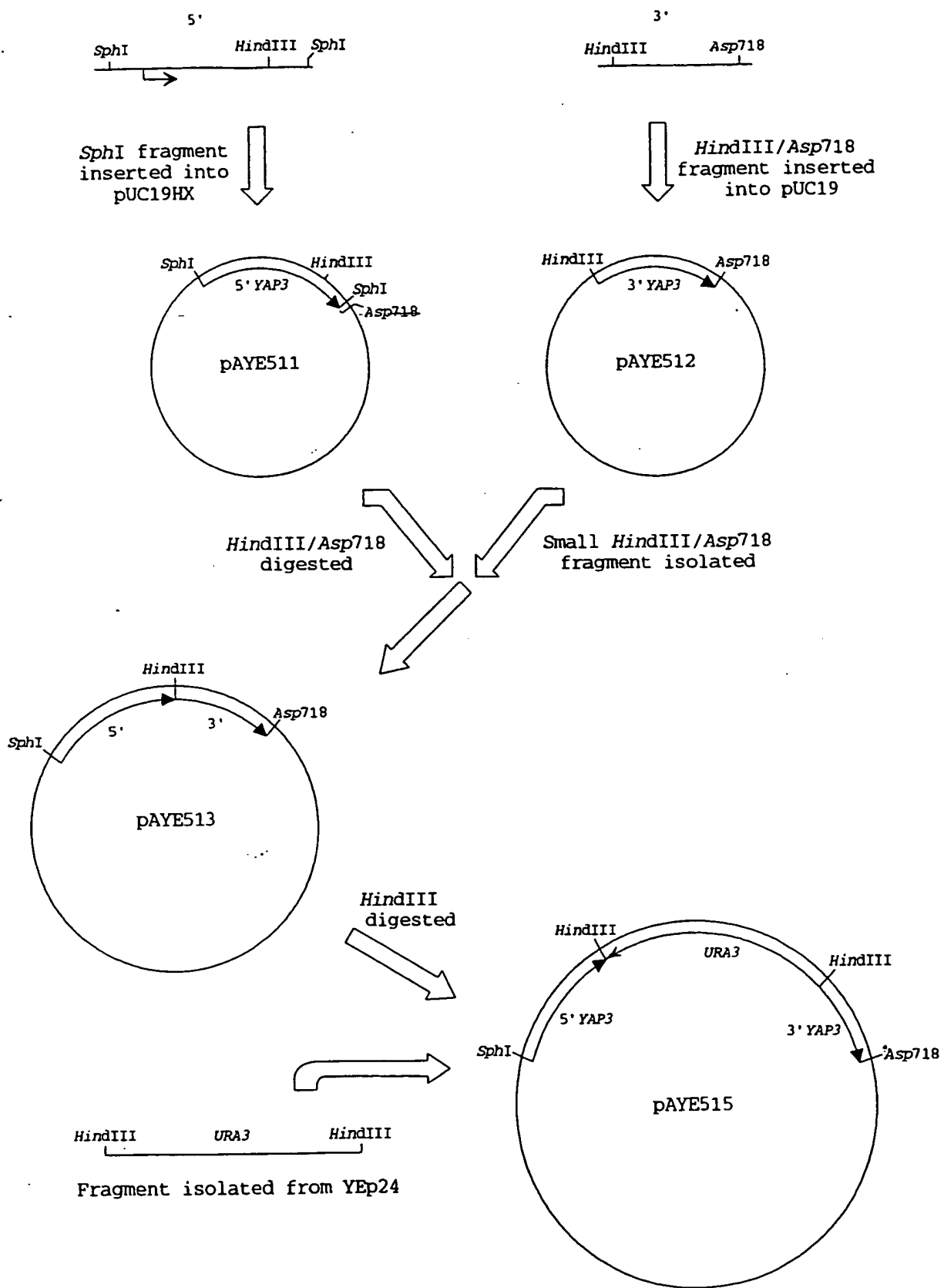
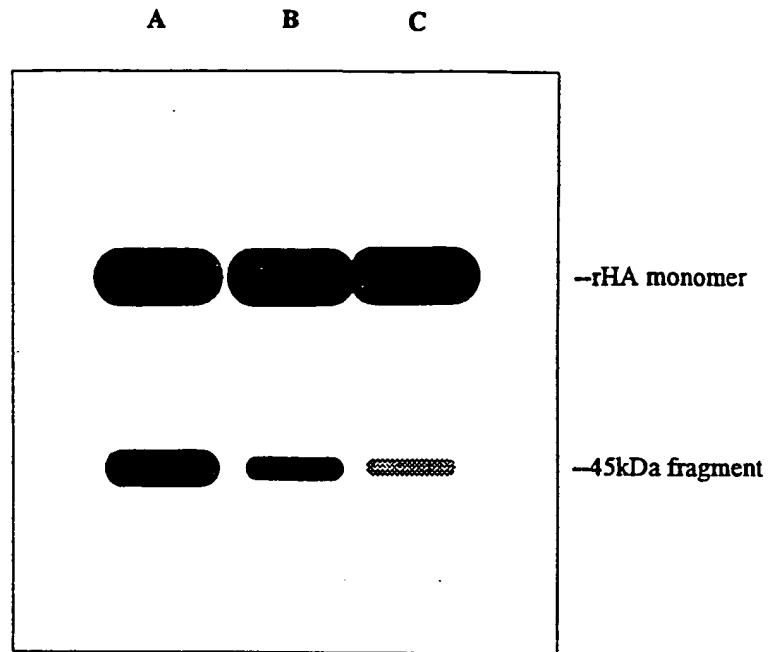
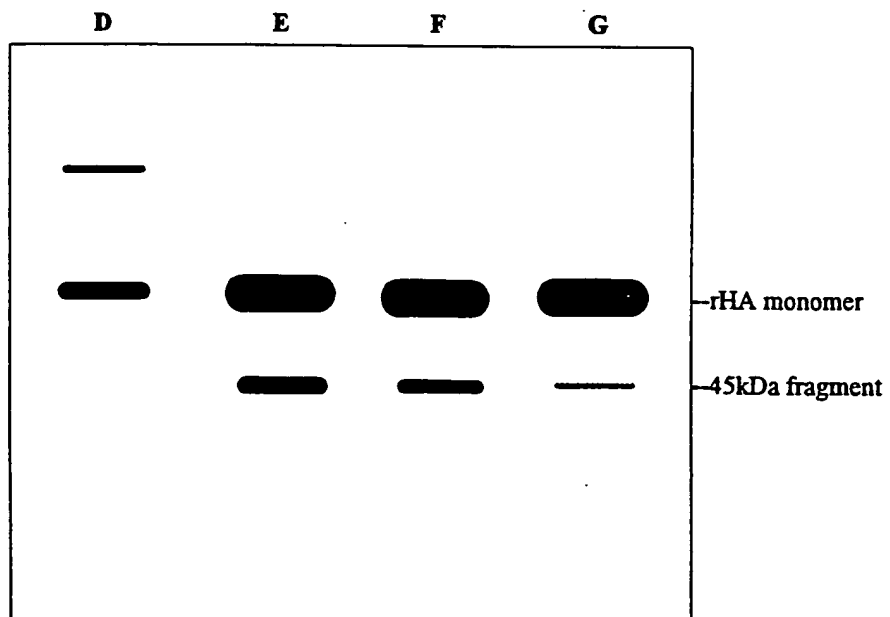
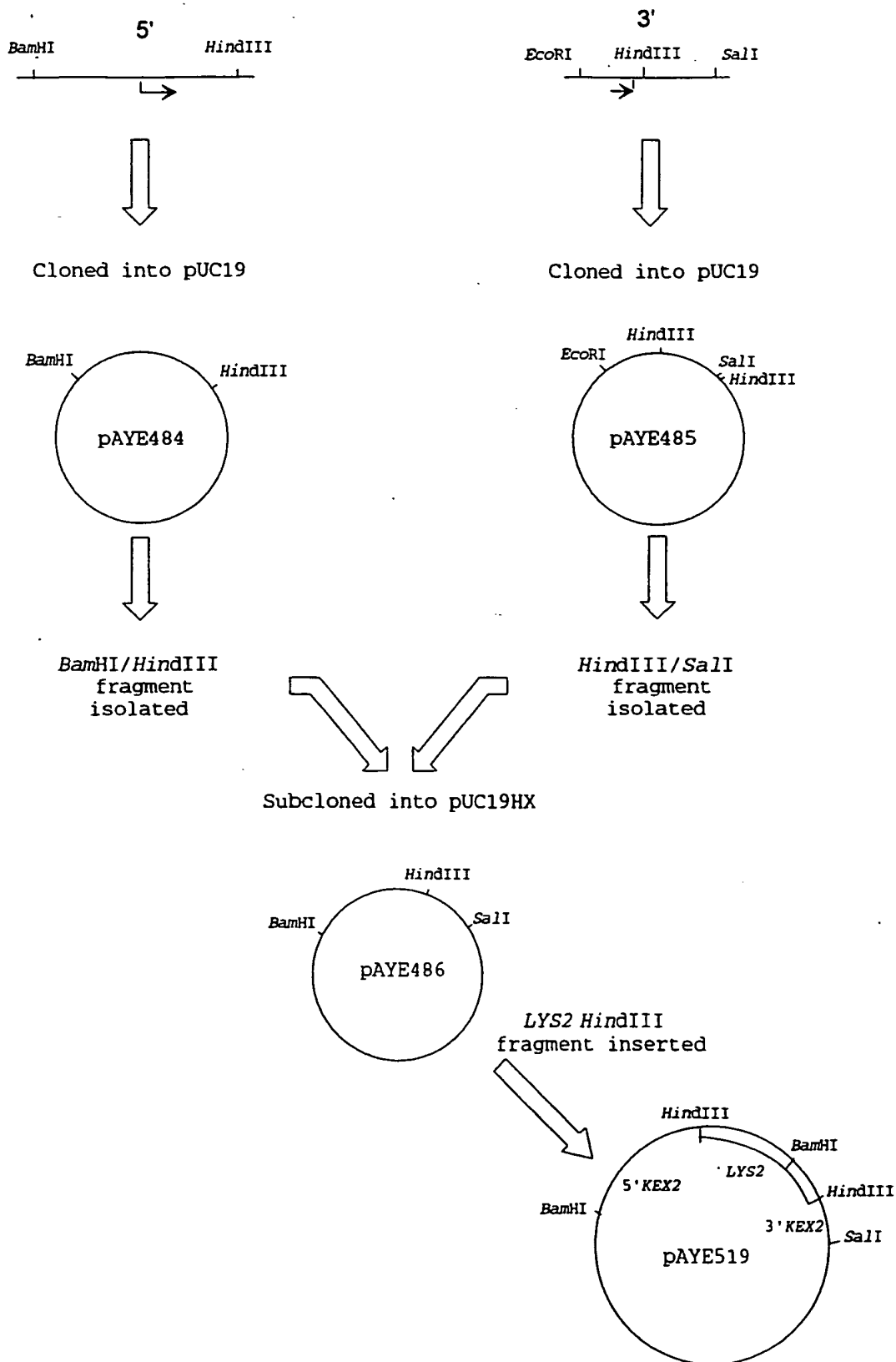


Figure 4Figure 5

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Figure 6

5' and 3' regions of KEX2 obtained by PCR:



A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/12 C12N1/21 A61K38/38 C12P21/02 C12N15/11
C12N1/20

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N A61K C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,A	BIOCHIMIE, vol. 76, 1994 pages 226-233, Y. BOURBONNAIS ET AL. 'Cleavage of prosomatostatins by the yeast Yap3 and Kex2 endoprotease' *see the whole article* ---	1-21
Y	BIOTECHNOLOGY, vol. 8, no. 1, 1990 pages 42-46, D. SLEEP ET AL. 'The secretion of human serum albumin from the yeast <i>S. cerevisiae</i> using five different leader sequences' *see the whole article* --- -/--	1-21

☒ Further documents are listed in the continuation of box C.

☐ Patent family members are listed in annex.

* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
 "E" earlier document but published on or after the international filing date
 "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
 "O" document referring to an oral disclosure, use, exhibition or other means
 "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
 "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
 "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
 "&" document member of the same patent family

Date of the actual completion of the international search

23 June 1995

Date of mailing of the international search report

- 5. 07. 95

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Fax (+ 31-70) 340-3016

Authorized officer

Marie, A

C(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EMBO JOURNAL, vol. 12, no. 1, 1993 pages 285-294, Y. BOURBONNAIS ET AL. 'Isolation and characterisation of S. cerevisiae mutants defective in somatostatin expression...' *see the whole article* ---	1-21
Y	YEAST, vol. 6, 1990 pages 127-137, M. EGEL-MITANI ET AL. 'A novel aspartyl protease allowing KEX2-independent MF alfa propheromone processing in yeast' *see the whole article* -----	1-21